Article

Immunotherapy of Epstein-Barr Virus Associated Malignancies Using Mycobacterial HSP70 and LMP2A$_{356-364}$ Epitope Fusion Protein

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Epstein-Barr virus infection is strongly associated with a number of malignancies. The EBV latent membrane protein 2A has been implicated as one of the most attractive candidates for immunotherapy of related malignancies. In previous studies, the T cell epitopes of LMP2A have been identified systematically. However, the epitope-based vaccine generally meets inefficient immunogenicity when used *in vivo* directly, which could be overcome by combination with appropriate adjuvants. Heat shock protein is a natural chaperon, which is able to activate the classical major histocompatibility complex class I antigen-processing pathway (cross-presentation). In this study, a minigene encoding LMP2A$_{356-364}$ (FLYALALLL) was genetically fused to the carboxy-terminal of mycobacterial heat shock protein 70. The epitope fusion protein was expressed and purified, and the cross-presentation of LMP2A$_{356-364}$ by monocyte-derived dendritic cells pulsed with the epitope fusion protein was evaluated. Results showed that the epitope fusion protein-pulsed mDCs were much more efficient than the single peptide-pulsed mDCs on CTL activation. Immunization of HLA-A2.1 transgenic mice with MtHsp70-LMP2A$_{356-364}$ generated peptide specific CTL more effectively than a single peptide plus incomplete Freund's adjuvant (IFA). Growth of LMP2A expressing B16 melanoma tumor cells was suppressed in the vaccinated groups. Our results suggested that MtHsp70-LMP2A$_{356-364}$ fusion protein was more effective than the CD8$^+$ T cell epitope alone on anti-tumor immunity. As a result, the MtHsp70-LMP2A$_{356-364}$ fusion protein is considered to be a promising candidate vaccine for EBV related malignancies. *Cellular & Molecular Immunology*. 2009;6(6):423-431.

Key Words: mycobacterial heat shock protein 70, Epstein-Barr virus, latent membrane protein 2A, epitope, cytotoxic T-lymphocytes

Introduction

Epstein-Barr virus (EBV) is a ubiquitous gammaherpesvirus that infects over 95% of the world's population and strongly associates with a number of malignancies. Nasopharyngeal carcinoma (NPC) is the most frequent EBV associated malignancy which is pandemic in Southern China and Southeast Asia (1). Previous studies have found that LMP2A is a promising target antigen in immunotherapy of NPC (2-7). Much work has been done to identify the epitopes of LMP2A presented by HLA-A2, A11, A23, A24, A25, B27, B60 and B63 (8-11). Furthermore, many clinical trials on LMP2A epitope based vaccines have been carried out in the past years. However, moderate or low responses have been observed in human trials and animal model tests (12-15). One reason might be the poor immunogenicity of the LMP2A derived peptides. On the other hand, LMP2A lacks MHC II-restricted epitopes and could not elicit CD4$^+$ T-helper 1 cell responses (16-18). Chimeric antigens containing both CD4$^+$ and CD8$^+$ T-cell epitopes have been designed to treat EBV-positive nasopharyngeal carcinoma, but they cannot induce CTL responses, which remains a major obstacle to

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Abbreviations: EBV, Epstein-Barr virus; LMP2A, latent membrane protein 2A; NPC, nasopharyngeal carcinoma; MtHsp70, mycobacterium tuberculosis heat shock protein 70; CTL, cytotoxic T lymphocyte; IFA, incomplete Freund's adjuvant; mDCs, Monocyte-derived dendritic cells; HSP, heat shock protein.

develop effective vaccine (19).

Mycobacterium tuberculosis HSP70（MtHsp70）is a powerful antigen which is rich in multiple B and T cell epitopes. Previous studies indicate that MtHsp70 can be used as an adjuvant-free carrier to stimulate the humoral and cellular immune response to accompanied proteins or synthetic peptides (20, 21). Many studies have demonstrated that MtHsp70 accompanied antigens are able to induce potential antigen-specific $CD8^+$ T cells with cytolytic and cytokine-secreting functions through $CD4^+$ T cell independent mode (22, 23).

To explore whether MtHsp70 can remedy the shortcoming of LMP2A in tumor immunotherapy, we genetically fused a minigene encoding the cytotoxic T lymphocyte (CTL) epitope LMP2A$_{356-364}$ and linker sequences (GSG) to the carboxyl terminal of MtHsp70, which was expressed in *E. coli.* BL21. The immune functions of the purified fusion protein were analyzed both in healthy EBV carrier donors and HLA-A2.1 transgenic mice. Our results indicated that compared to the effect of the $CD8^+$ T cell epitope alone, the fusion protein containing LMP2A $CD8^+$ T cell epitopes and MtHsp70 enhanced tumor immunity, indicating that fusion protein of the MtHsp70 and LMP2A antigen epitope was a promising candidate as a cancer vaccine.

Materials and Methods

Transgenic mice and cell lines

C57BL/6-Tg(HLA-A2.1)1Enge/J transgenic homozygous mice were purchased from The Jackson Laboratory (strains: 003475) through National Resource Centre for Mutant Mice (Nanjing University, Nanjing 210061, China) and were subsequently bred in the specific pathogen free animal centre of Nanjing Medical University. All mouse studies were approved by Animal Care and Use Committee of Nanjing Medical University.

The B16-F10 melanoma cell line (B16, C57BL/6 origin) was presented by Dr. Jun Dou (Southeast University, Nanjing 210009, China). B16-HLA-A2.1-LMP2A cell (HLA-A2.1+, LMP2A+) co-expressing HLA-A2.1 and LMP2A molecules was constructed by transfecting B16 cell with plasmid pIRES-HLA-A2.1-LMP2A previously. B16 cell was cultured in RPMI 1640 (Gibco, USA) supplemented with 10% heat-inactivated fetal calf serum (Sijiqing, Hangzhou, China), 2 mM L-glutamine, 5 mM 2-mercaptoethanol, 100 μg/ml penicillin and streptomycin; B16-HLA-A2.1-LMP2A cell was cultured in media supplemented with 1000 μg/ml G418 (Gibco, USA); T2 (HLA-A2.1+, LMP2A-) cell and specific peptide-loaded T2 cell, PBMCs and monocyte- derived dentritic cells (mDCs) used in this study were described in our previous works (8, 24).

Recombinant proteins and peptides

MtHsp70 recombinant expression vector pET28a-MtHsp70 was constructed and expressed previously. In brief, the gene encoding mycobacterium tuberculosis heat shock protein 70 (MtHsp70) was amplified from genomic DNA of standard strains H37Rv (ATCC, Manassas, VA) by PCR. The PCR product (1896 bp) was digested by BamH I and Xho I and then cloned into the expressing vector pET-28a(+) (Novagen, USA). Then the plasmid pET28a-MtHsp70 was used as template, the coding sequence of LMP2A$_{356-364}$ was ligated to the carboxyl-terminus of MtHsp70 with a linker (GSG) sequence by extended PCR using the forward primer 5'-GGC GGATCC ATG GCT CGT GCG GTC GGG ATC-3' (underline: BamH I site) and the reverse primer 5'-CCG CTCGAG TCA **CAA CAA GAG TGC GAG AGC ATA TAG GAA** *CCC GGA CCC* CTT GGC CTC-3' (underline, Xho I site; bold, LMP2A$_{356-364}$ coding sequence; Italic: linker sequence). The resultant PCR product was sub-cloned into the pET28-a(+) expressing vector to yield MtHsp70-LMP2A$_{356-364}$ expression plasmid pET28a-MtHsp70-LMP2A$_{356-364}$ (Figure 1). The recombinant epitope-fusion-protein MtHsp70-LMP2A$_{356-364}$ was expressed in *E. coli* BL21 (DE3) and purified by Ni^{2+}-NTA affinity chromatography column (Sigma-Aldrich, Saint Louis, Missouri, USA). Contaminated endotoxin of MtHsp70 and MtHsp70-LMP2A$_{356-364}$ preparations was removed by passing through Detoxi-Gel Endotoxin Removal Gel (Pierce, Rockford, IL, USA) for three times. Endotoxin level was determined by quantitative limulus amoebocyte lysate (LAL) assay（Xmhsj, Xiamen, China）and controlled containing less than 0.05 EU/mg.

LMP2A derived peptide LMP2A$_{356-364}$ (FLYALALLL) was synthesized according to standard F-moc solid phase synthesis methods (GenScript, Nanjing, China). Peptide purity was determined by analytical reverse-phase HPLC, and purity was routinely > 95%. Lyophilized peptides were diluted in DMSO and stored at -20 ºC.

Generation LMP2A$_{356-364}$ epitope specific CTL in vitro

Autologous mDCs of EBV seropositive and HLA-A2.1 type matched normal donors were induced according to previous report (25). Cultured for 5 days, mDCs were harvested, stimulated with 10 μg/mL MtHsp70-LMP2A$_{356-364}$, MtHsp70 and LMP2A$_{356-364}$ peptide for 4 h, and then washed twice in serum-free RPMI 1640. Peripheral blood lymphocytes (2×10^6) and protein/peptide-pulsed autologous mDCs (2×10^5) were co-cultured in 1 mL RPMI 1640 supplemented with 10% FCS in 24-well plates. The cells were restimulated with fresh protein/peptide-pulsed autologous dendritic cells every 7 days for two times. On day 3 after first stimulation, 20 IU/ml recombinant human IL-2 (Peprotech, England) was supplemented. Media were changed every 3 days with half-fresh medium in the presence of recombinant human IL-2. On day 7 after the last stimulation, cells were harvested and LMP2A$_{356-364}$ epitope specific cytolytic activity of T lymphocytes was measured using two-color fluorescence assay as described in our previous work (8).

Mice vaccination and generation of CTL

HLA-A2.1 transgenic mice, 6 to 10 weeks old, were divided into 3 groups (each group containing 5 mice). Group 1 to Group 3 were immunized with 200 μg MtHsp70, MtHsp70-

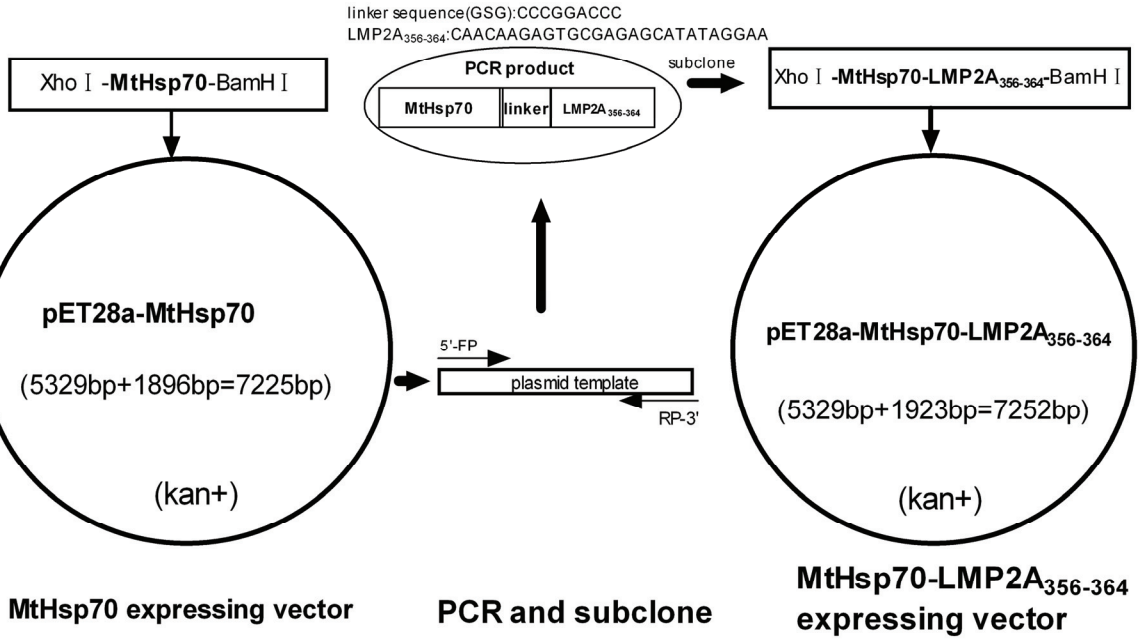

Figure 1. Flowchart of construction of expression vectors of MtHsp70-LMP2A$_{356-364}$ fusion protein. Plasmid pET28a-MtHsp70 containing the full gene encoding mycobacterium tuberculosis heat shock protein 70 was carried out as amplify template. Extended PCR with reverse primer containing linker sequences and LMP2A$_{356-364}$ epitope encoding sequences was used to construct the mini gene MtHsp70-linker-LMP2A$_{356-364}$. The PCR product of the mini gene was purified and subcloned into a pET-28a(+) empty vector by Xho Ⅰ and BamH I restricted enzyme sites.

LMP2A$_{356-364}$, 20 μg LMP2A$_{356-364}$ emulsified in incomplete Freund's adjuvant (IFA) respectively. Adjust the vaccinate volume to 200 μl/mouse. Mice were subcutaneously immunized for three times (days 0, 7, 14). Seven days after final immunization, the mice were sacrificed and splenocytes were re-suspended at 1 × 10^6/ml and stimulated with 10 μg/ml MtHsp70, MtHsp70 LMP2A$_{356-364}$ and LMP2A$_{356-364}$ for 5 days respectively.

IFN-γ ELISPOT assay

Mouse IFN-γ ELISPOT assay was performed in PVDF bottomed 96-well plates (Millipore, USA) by using a murine IFN-γ ELISPOT kit (eBioscience, USA) according to the manufacturer's instructions. Re-stimulated splenocytes (2.5 × 10^5/well) were then added together with the lethally irradiated B16-HLA-A2.1-LMP2A and B16 cells (5 × 10^4/well) respectively. Positive (5 μg/ml ConA), negative (coated without anti-IFN-γ capture antibody) and background (culture media) control wells were also conducted. Following steps were performed as described anywhere (8, 26). Finally, the spots were counted by ImmunoSpot Analyzer (CTL, Beijing, China). In this study, the averages of the spots were expressed as the number of spot form cells (SFC)/2.5 × 10^5 $CD8^+$ T cells.

Cytotoxicity assay

The CytoTox 96 nonradioactive cytotoxicity assay (Promega, USA) was performed to measure the cytotoxic activity of the restimulated splenocytes according to the manufacturer's instructions. Briefly, 1 × 10^5/well B16-HLA-A2.1-LMP2A and B16 cells were added to 96-well uncoated plates (Costar, USA) as target cells. Re-stimulated splenocytes were added at various effector-to-target ratios (E:T = 40:1, 20:1, 10:1, 5:1 respectively) as effector cells. Control groups of effector cells spontaneous LDH release, target cells spontaneous LDH release, target cells maximum LDH release, volume correction control and culture medium background were performed at the same time. The plates were incubated for 4 hours in a humidified chamber at 37 ºC, 5% CO_2, then centrifuged at 500 g for 5 min. Aliquots (50 μl) were transferred from all wells to fresh 96-well flat-bottom plates, and an equal volume of reconstituted substrate mix was added to each well. The plates were incubated at RT for 30 min and protected from light. Then 50 ml stop solution was added, and the absorbance values were measured at 492 nm. The mean percentage of specific lysis in triplicate wells was calculated as follows: % Cytotoxicity = (Experimental – Effector Spontaneous – Target Spontaneous) / (Target Maximum – Target Spontaneous) ×100

Tumor prophylactic assay

HLA-A2.1 transgenic mice were immunized with the appropriate preparations (MtHsp70, MtHsp70-LMP2A$_{356-364}$ and IFA-LMP2A$_{356-364}$) as described above. Following the last injection (day 0), mice were inoculated by s.c. injection on the right flank with 1 × 10^6 B16-HLA-A2.1- LMP2A cells. Mice were scarified when the tumor grew up to 400 mm^2 according to animal ethics committee. Each group consisted

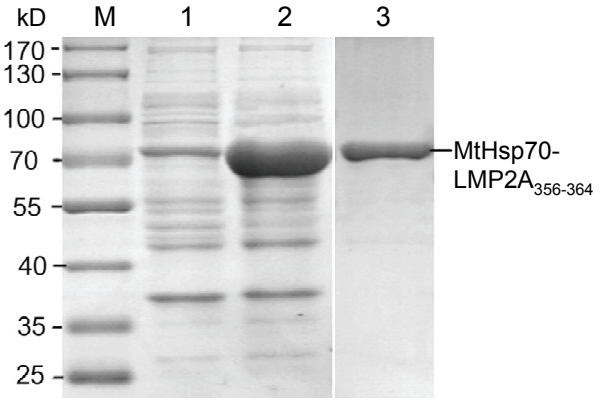

Figure 2. SDS-PAGE analysis for the expression and purification of MtHsp70-LMP2A$_{356-364}$ epitope fusion protein. Lane M, Pre-stained protein molecular ladder; Lane 1, pET28a-MtHsp70-LMP2A$_{356-364}$/BL21(DE3) cultured at 37ºC for 4 h without IPTG induction (control); Lane 2, pET28a-MtHsp70-LMP2A$_{356-364}$/BL21(DE3) cultured in 37ºC for 4 h with 0.4 mmol/L IPTG induced expression; Lane 3, MtHsp70-LMP2A$_{356-364}$ preparations purified with Ni^{2+}-NTA His Bind Resin affinity column.

of 5 mice and the experiments were repeated twice.

Tumor therapeutic assay

HLA-A2.1 transgenic mice were inoculated intradermally with 1×10^6 B16-HLA-A2.1-LMP2A cells on the right flank. Two weeks after tumor transplant, tumors were palpable (4-5 mm in diameter) and the mice were randomly divided into 3 groups (5 mice per group). Each group of mice was subcutaneously immunized for three times (days 14, 21, 28) with MtHsp70, MtHsp70-LMP2A$_{356-364}$ and IFA-LMP2A$_{356-364}$ respectively as described previously. Tumors were measured with calipers and the products of perpendicular diameters were recorded. Mice were killed once tumors reached 400 mm^2. All experiments were performed independently at least twice with similar results.

Statistical analysis

Unless noted, data were presented as mean ± SD. ANOVA Newman-Keuls Multiple Comparison Test was used for statistical analyses to compare the differences among multiple groups. The unpaired two-tailed Student's *t*-test was used to analyze differences between two groups. Differences were considered significant for *p* values less than 0.05. Survival analysis was estimated by the Kaplan-Meier method. All statistical analyses were performed with the SPSS 13.0 software.

Results

MtHsp70-LMP2A$_{356-364}$ expression and purification

pET28a-MtHsp70-LMP2A$_{356-364}$ was constructed and transformed into *E. coli* BL21(DE3) strain. Expression of MtHsp70-LMP2A$_{356-364}$ was induced at cell exponential phase with 0.4 mM isopropyl-β-d-thiogalactoside (IPTG) and cell growth continued for another 4 h at 37ºC before harvesting. The 70 kd overproduced soluble fusion protein MtHsp70-LMP2A$_{356-364}$ was purified by Ni^{2+}-NTA affinity chromatography columns (Figure 2). The purified protein was passed through the Detoxi-Gel Endotoxin Removal Gel for three times in order to remove contaminated endotoxin. Approximately 10 mg endotoxin free (0.042 EU/mg) MtHsp70-LMP2A$_{356-364}$ was recovered with one liter of initial bacterial broth. The endotoxin level of Endotoxin Removal Gel treated MtHsp70 and MtHsp70-LMP2A$_{356-364}$ was 0.038 and 0.042 EU/mg respectively (Data of MtHsp70 expression and purification not shown), while untreated MtHsp70 and MtHsp70-LMP2A$_{356-364}$ were 0.431 and 0.571 EU/mg respectively.

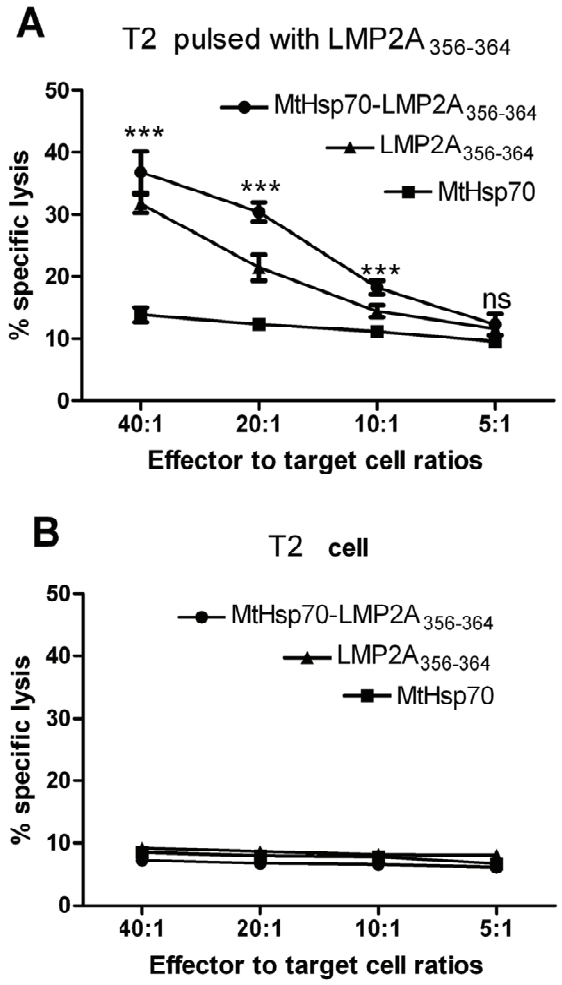

Figure 3. Fusion protein MtHsp70-LMP2A$_{356-364}$ elicited LMP2A$_{356-364}$ specific cytotoxicity *in vitro*. LMP2A$_{356-364}$ synthetic peptide-pulsed T2 cells (A) and naïve T2 cells (B) were used as target cells. PBMCs stimulated by autologous mDCs pulsed with MtHsp70-LMP2A$_{356-364}$ fusion protein, LMP2A$_{356-364}$ synthetic peptide and MtHsp70 were used as effect cells. The E:T ratios were 5:1, 10:1, 20:1 and 40:1 for each group. The data represent the means and standard errors of three separate health donors.

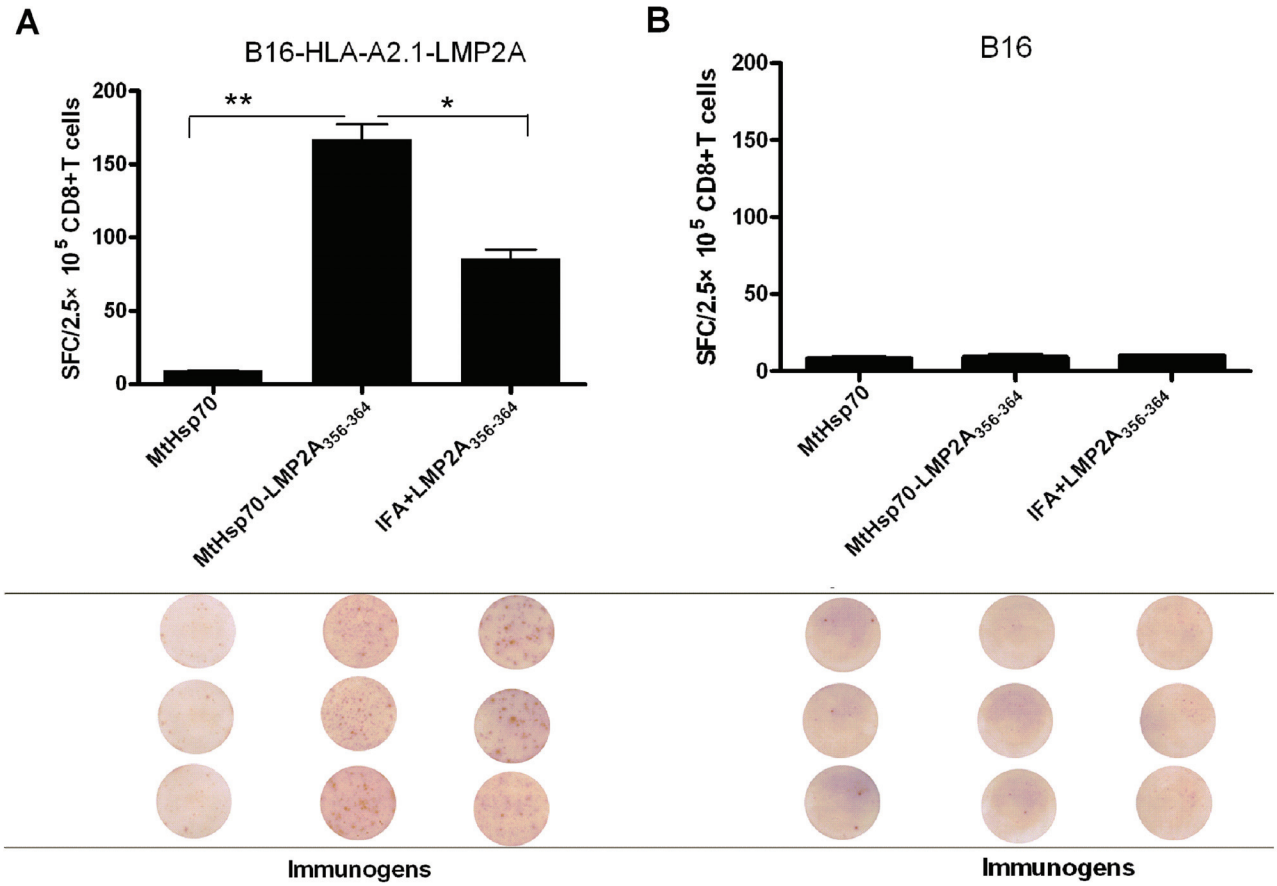

Figure 4. Increased IFN-γ production of CTLs in HLA-A2.1 transgenic mice immunized with MtHsp70-LMP2A$_{356-364}$. HLA-A2.1 transgenic mice were immunized with MtHsp70, MtHsp70-LMP2A$_{356-364}$ and IFA+LMP2A$_{356-364}$ respectively. IFN-γ ELISPOT assay was carried out according to the protocol. The spots were counted by ImmunoSpot Analyzer. Results were demonstrated as the number of spot-forming cells (SFC) per 2.5 × 10^5 CTL. (A) Restimulated splenocytes (2.5 × 10^5 cells/well) were added together with the lethally irradiated (50 Gy) B16-HLA-A2.1-LMP2A (5 × 10^4 cells/well). (B) Restimulated splenocytes (2.5 × 10^5 cells/well) were added together with the lethally irradiated (50 Gy) B16 cells (5 × 10^4 cells/well) (n = 5).

MtHsp70-LMP2A$_{356-364}$ elicited LMP2A$_{356-364}$ specific cytolytic activity in health donors

Epitope specific cytotoxiciy assay was carried out to determine whether the recombinant fusion protein MtHsp70-LMP2A$_{356-364}$ was able to induce a specific T lymphocyte response. According to the results shown in Figure 3, the CTLs induced by MtHsp70-LMP2A$_{356-364}$ were able to lyse the LMP2A$_{356-364}$ peptide-pulsed T2 cells but failed to lyse the naïve T2 cells. Moreover, CTLs induced by MtHsp70-LMP2A$_{356-364}$ demonstrated much stronger (p = 0.003) cytotoxicity than CTLs induced by LMP2A$_{356-364}$ peptide. The CTLs elicited by MtHsp70 showed specific cytolytic activity to neither LMP2A$_{356-364}$ peptide-pulsed T2 cells nor naïve T2 cells.

MtHsp70-LMP2A$_{356-364}$ elicited LMP2A specific IFN-γ secretion and cytotoxicity of CTLs in transgenic mice

To determine the effects of the recombinant fusion protein MtHsp70-LMP2A$_{356-364}$ on CTLs, HLA-A2.1 transgenic mice were immunized with MtHsp70-LMP2A$_{356-364}$, MtHsp70 and LMP2A$_{356-364}$ peptide. CTLs of HLA-A2.1 transgenic mice were isolated and re-stimulated with the lethally irradiated B16-HLA-A2.1-LMP2A and B16 cells (5 × 10^4/well) respectively.

LMP2A expressing tumor cells B16-HLA-A2.1-LMP2A, but not B16 cells were able to induce IFN-γ secretion of the CTLs from HLA-A2.1 transgenic mice (Figure 4). Moreover, in B16-HLA-A2.1-LMP2A stimulation group, CTLs sensitized by MtHsp70-LMP2A$_{356-364}$ demonstrated the highest level of IFN-γ secretion, which was significantly higher than LMP2A$_{356-364}$ and MtHsp70 according to IFN-γ ELISPOT assay (Figure 4A).

To learn the cytolytic activity of CTLs, a non-radioactive cytotoxicity assay was performed with the target cells B16-HLA-A2.1-LMP2A (Figure 5A) and B16 (Figure 5B). CTLs isolated from HLA-A2.1 transgenic mice demonstrated strong cytotoxicity to B16-HLA-A2.1-LMP2A cells, while no obvious specific cytotoxicity was observed on B16 target cells (Figure 5). In the cytotoxicity assay system CTL: B16-HLA-A2.1-LMP2A (E:T), CTLs of MtHsp70-LMP2A$_{356-364}$

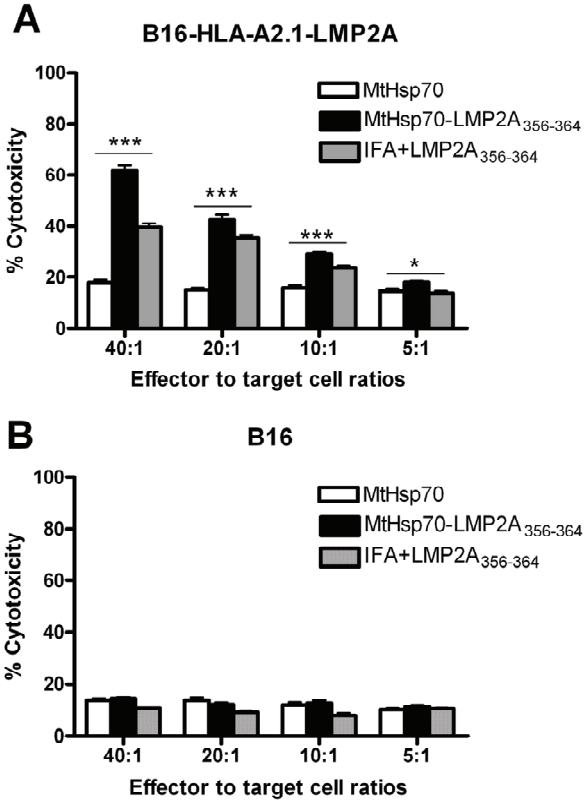

Figure 5. Cytotoxicity assay of CTL elicit in HLA-A2.1 transgenic mice. B16-HLA-A2.1-LMP2A (A) and B16 (B) cells were set as target cells (1×10^5/well). Restimulated splenocytes were added at various effector-to-target ratios (E:T = 40:1, 20:1, 10:1, 5:1 respectively) as effector cells. Cytotoxicity assay of CTL was performed with the CytoTox 96 non-radioactive method. The results from three independent experiments were shown as mean ± SD.

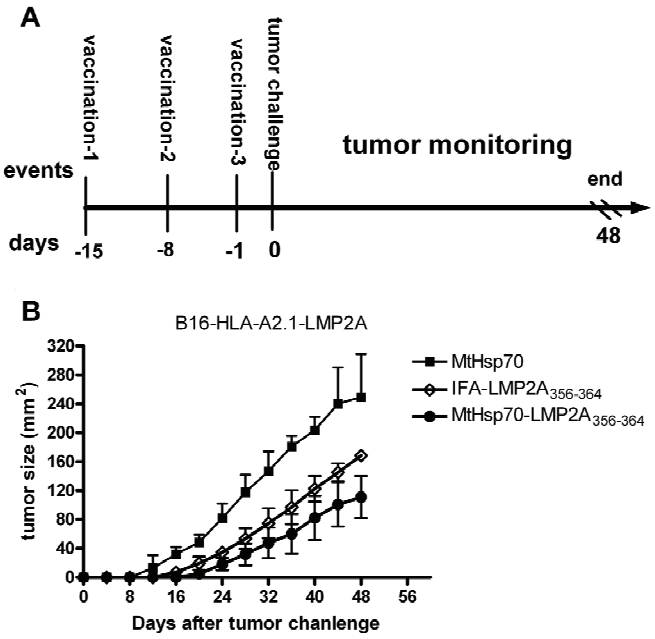

Figure 6. Pre-immunization with recombinant MtHsp70-LMP2A$_{356-364}$ fusion protein inhibited B16-HLA-A2.1-LMP2A growth. HLA-A2.1 transgenic mice were immunized subcutaneously with MtHsp70, IFA-LMP2A$_{356-364}$, and MtHsp70-LMP2A$_{356-364}$. After the final immunization, mice were challenged subcutaneously with 1×10^6 B16-HLA-A2.1-LMP2A tumor cells to the right flank area. Tumor size was monitored every 4 days. Each group contained 5 mice and the experience repeated at least twice. (A) Flowchart of pre-immunization and tumor challenge. (B) Tumor growth curves of each group. Data refer to the tumors size with mean ± SD per group.

immunization group demonstrated a significantly stronger cytotoxicity than IFA-LMP2A$_{356-364}$ group. As a negative control, MtHsp70 demonstrated no immunogenicity in eliciting LMP2A specific CTL response (Figure 5A).

MtHsp70-LMP2A$_{356-364}$ induced anti-tumor immunity

In vivo anti-tumor immunity was assessed in HLA-A2.1 transgenic mice with B16-HLA-A2.1-LMP2A challenge. Compared with MtHsp70 immunization group, mice immunized with MtHsp70-LMP2A$_{356-364}$ or IFA-LMP2A$_{356-364}$ showed significant tumor growth inhibitory effects, and the inhibitory effect of MtHsp70-LMP2A$_{356-364}$ was stronger than IFA-LMP2A$_{356-364}$ (Figure 6), indicating that LMP2A was able to induce a significant anti-tumor immunity, which was promoted by MtHsp70.

In mice survival analysis, 60% of mice in MtHsp70-LMP2A$_{356-364}$ immunization group survived for over 60 days after B16-HLA-A2.1-LMP2A tumor challenge, 60% mice in IFA-LMP2A$_{356-364}$ immunization group died within 60 days, and all mice in MtHsp70 immunization group died within 44 days (Figure 7). These results indicated that fusion protein MtHsp70-LMP2A$_{356-364}$ was able to induce an anti-tumor immunity against LMP2A expressing tumor cells *in vivo*.

Discussion

EBV associated malignancies such as NPC and Hodgkin's lymphoma cells usually express EBNA-1, LMP1, and LMP2. LMP1 is not a dominant immunogenic protein and LMP1-specific CTLs are uncommon. EBNA-1 is rich in glycine-alanine repeat region that prevents proteosome-dependent degradation and HLA class I mediated peptide presentation. EBNA-1 specific CTLs cannot lyse the EBV associated tumor cells effectively. LMP2A specific CTLs are a subdominant population of CTLs elicited by EBV infection *in vivo*, so LMP2A is an ideal target antigen for immunotherapy of EBV associated malignancies (27-30). Owing to the advantages of peptide-based vaccine, many studies have focused on LMP2A epitope-based vaccines and a lot of HLA restricted T cell epitopes of LMP2A have been identified (8, 10, 31-33). Moreover, there is a growing emphasis on enhancing the immunogenicity of peptide-based vaccine, such as development of effective human adjuvants and peptide vaccine delivery systems (34). Previous clinical trials

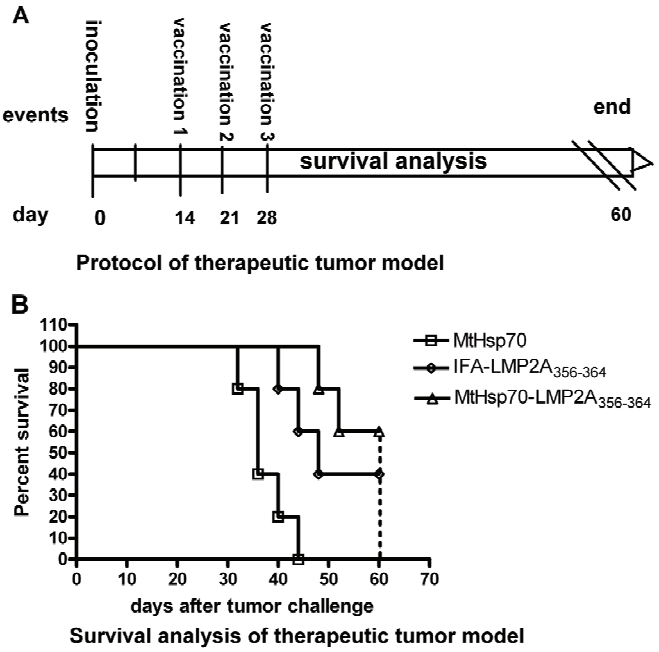

Figure 7. Therapeutic immunization with the MtHsp70-LMP2A$_{356-364}$ fusion protein leads to long-term survival after B16-HLA-A2.1-LMP2A tumor challenge. HLA-A2.1 transgenic mice (6-8 week) were divided into three groups (n = 5). Each mouse was injected subcutaneously with 1×10^6 B16-HLA-A2.1-LMP2A tumor cells in the right flank area. (A) Therapeutic vaccinations program. (B) Survival mice of each group were documented every 4 days after B16-HLA-A2.1-LMP2A tumor challenge.

and animal experiments have used oil-emulsion-type adjuvant, but this traditional adjuvant has shown serious side effects (12, 15). Here we have produced a fusion protein containing a conserved LMP2A epitope and the MtHsp70, which offers a strategy to elicit the anti-tumor immunity of specific CTLs. This study has shown that immunization with the MtHsp70-LMP2A$_{356-364}$ epitope fusion protein in the absence of adjuvant is capable of providing stronger protection to HLA-A2.1 transgenic mice against challenge with B16-HLA-A2.1-LMP2A cells than that of IFA-LMP2A$_{356-364}$. It should be a new deliver system for epitope-based vaccine.

Heat shock proteins (HSP) derived from cancer cells and virus-infected cells are able to elicit tumor-specific or virus-specific immunity (35). Several clinical trials in different tumor types have been conducted using this vaccination strategy. Gp96 is an autologous tumor-derived heat shock protein, a recent phase III clinical trial has demonstrated that gp96 peptide complex vaccine is partially effective in curing M1a and M1b melanoma patients (36). Our study indicates that LMP2A epitope covalently conjugated to the carboxyl terminal of MtHsp70 shows immunological activity *in vitro* and *in vivo*. It has two advantages compared with traditional HSP-based vaccines. First, recombinant HSP-epitope fusion protein eliminates the quantity limitation of autologous tumor-derived HSP-peptide complex, especially in Hodgkin's lymphoma and NPC. Surgery is seldom selected in treating these malignancies and few self tumor tissues are available to extract autologous tumor-derived HSP-peptide complex (37). Second, a recombinant exogenous heat shock protein, which replaces autologous heat shock protein, also acts as a Th1 polarizing adjuvant, which makes HSP-based vaccines more economical and feasible.

Tumor specific $CD4^+$ and $CD8^+$ T cells have been considered as critical components in anti-tumor immunity. A successful tumor immunotherapy strategy includes induction of both $CD4^+$ and $CD8^+$ T cell immune responses against tumor cells (16, 38). Human $CD4^+$ T-helper 1 cell responses to Epstein-Barr virus (EBV) infection are also important in the maintenance of virus-specific memory $CD8^+$ T cells and antiviral effectors. However, among EBV latent-cycle proteins, $CD4^+$ T responses to LMP2A epitopes are much less frequent than EBNA1 and LMP1 (18). Previous studies have demonstrated that MtHsp70 accompanied antigens can induce potential antigen-specific $CD8^+$ T cells with cytolytic and cytokine-secreting functions through $CD4^+$ T cell independent modes (21, 23, 39-42). In this study, we conjugated a conserved LMP2A HLA-A2.1 restricted epitope LMP2A$_{356-364}$ to MtHsp70, which not only induces LMP2A$_{356-364}$ specific immune recognition *in vitro*, but also elicits LMP2A specific CTL *in vivo*. These results indicate that the fusion protein can be successfully processed and cross-presented by antigen present cell both *in vitro* and *in vivo*.

In summary, our studies show that adjuvant free immunization of HLA-A2.1 transgenic mice with an MtHsp70-LMP2A$_{356-364}$ epitope fusion protein is effective in the prophylaxis and therapy of an LMP2A expressing malignancies. Mice immunized with the MtHsp70-LMP2A$_{356-364}$ fusion protein are superior to the traditional IFA adjuvant synthetic peptides in survival rate analysis. All of these indicate that MtHsp70 and LMP2A reconstituted epitope fusion protein may be a new strategy to enhance the potency of LMP2A epitope based vaccine in related tumor immunotherapy.

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